ANTI-COCAINE CATALYTIC ANTIBODY

Background of the Invention

Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding Sequence Listing and the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Catalytic antibodies have unique potential for the treatment of cocaine addiction and overdose. Cocaine reinforces self-administration by inhibiting a dopamine re-uptake transporter (1) in the mesolimbocortical "reward pathway". No antagonist to cocaine is known (2), perhaps reflecting the difficulties inherent in blocking a blocker. As an alternative to receptor-based therapeutics, a circulating agent could interrupt the delivery of cocaine to its binding site in the brain (3). An agent such as an antibody that merely bound the drug could be depleted stoichiometrically by complex formation but an enzyme that bound drug, transformed it and released product would be available for additional binding. Catalytic antibodies, a novel class of artificial enzyme, are inducible for a wide array of reactions and their substrate specificity is programmable to small molecules such as cocaine (4).

Cocaine detoxification is particularly well suited for a catalytic antibody approach. First, hydrolysis of the benzoyl ester of cocaine yields the biologically inactive products (5) ecgonine methyl ester and benzoic acid

(Figure 1). The plasma enzyme butyrylcholinesterase deactivates cocaine in humans (6) by means of this reaction. Second, acyl hydrolysis is the best studied of all antibody-catalyzed transformations (7,8). Esterase activity approaching that of natural enzymes has been reported (7) for catalytic antibodies and the large hydrophobic surface of the benzoyl ester is particularly well suited to elicit antibodies with strong binding and catalysis.

It has previously described (9) the first catalytic antibodies to degrade cocaine, Mab 3B9 and Mab 6A12. The antibodies were elicited by an immunogenic conjugate (TSA 1) of a phosphonate monoester transition-state analog (Scheme 1). The rate acceleration of these first artificial cocaine esterases (10^2-10^3) corresponded in magnitude to their relative stabilization of the ground-state to the transition-state (~ K_m/K_i). Catalytic antibodies with more potent catalytic mechanisms and with higher turnover rates are possible and, it has been estimated, necessary for clinical applications. Increased activity can be pursued either through repeated hybridoma generation or through mutagenesis of catalytic antibodies in hand. However, sequencing of the variable domains of Mab's 3B9 and 6A12 revealed 93% homology at the complementarity determining regions (see below). Such a lack of diversity has been noted previously for catalytic antibodies (10) and limits the opportunities for improving activity since a particular class of homologous catalytic antibodies may fail to optimize to the desired activity. A potential solution to this problem, that would not compromise the core structure of the analog, would be to vary the surfaces of the analog rendered inaccessible by attachment to carrier protein and thereby present distinct epitopes for immunorecognition.

The syntheses of three analogs of cocaine hydrolysis with identical phosphonate replacements but differing constructions for the immunoconjugates is now reported. The kinetics and the structural diversity of the catalytic antibodies elicited by these analogs has been characterized. The preferred catalytic antibodies for mutagenesis studies have been identified.

Summary of the Invention

The following standard abbreviations are used throughout the specification to indicate specific amino acids:

E represents Glutamic acid
S represents Serine
R represents Arginine
G represents Glycine
T represents Threonine
I represents Isoleucine
N represents Asparagine
Y represents Tyrosine
C represents Cysteine
P represents Proline
L represents Leucine
W represents Tryptophan
H represents Histidine
D represents Aspartic Acid
F represents Phenylalanine
Q represents Glutamine
V represents Valine
K represents Lysine
M represents Methionine
A represents Alanine
X represents any amino acid

The invention provides catalytic antibody capable of degrading cocaine characterized by comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is RSSXGTITXXNYAN (Seq ID No: 73), the amino acid sequence of complementarity determining region 2 is XNNYRPP (Seq ID No: 74) and the amino acid sequence of complementarity determining region 3 is ALWYSNHWV (Seq ID No: 75) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is DYNMY (Seq ID No: 76), the amino acid sequence of

complementarity determining region 2 is YIDPXNGXXFYNQKFXG (Seq ID No. 78) and the amino acid sequence of complementarity determining region 3 is GGGLFAX (Seq ID No: 78), wherein X can be any amino acid.

The present invention also provides a catalytic antibody capable of degrading cocaine comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is RSSSGTITANNYGS (Seq ID No. 40), the amino acid sequence of complementarity determining region 2 is VSNNRGP (Seq ID No: 41) and the amino acid sequence of complementarity determining region 3 is ALWNSNHFV (Seq ID No: 42) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is TYYIY (Seq ID No: 67), the amino acid sequence of complementarity determining region 2 is GMNPGNGVTYFNEKFKN (Seq ID No: 68) and the amino acid sequence of complementarity determining region 3 is VGNLFAY (Seq ID No: 69).

The present invention also provides a catalytic antibody capable of degrading cocaine comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is RSSXSLLYXDGKTYLN (Seq ID No: 79), the amino acid sequence of complementarity determining region 2 is LMSTRXS (Seq ID No: 80) and the amino acid sequence of complementarity determining region 3 is QXFXXYPFT (Seq ID No: 81) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is SDYAWX (Seq ID No: 82), the amino acid sequence of complementarity determining region 2 is YIRXXXXTRYNPSLXS (Seq ID No: 83) and the amino acid sequence of complementarity determining region 3 is XHYYGXXX (Seq ID No: 84).

The present invention provides a catalytic antibody capable of degrading cocaine comprising a light chain

wherein the amino acid sequence of complementarity determining region 1 is KSSQSLLYSDGKTYLN (Seq ID: 44), the amino acid sequence of complementarity determining region 2 is LVSKLDS (Seq. ID: 45) and the amino acid sequence of complementarity determining region 3 is VQGYTFPLT (Seq ID: 46) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is DHWMH (Seq ID: 71), the amino acid sequence of complementarity determining region 2 is TIDLSDTYTGYNQNFKG (Seq ID: 72) and the amino acid sequence of complementarity determining region 3 is RGFDY (Seq ID: 73).

In another embodiment, the present invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence RSSXGTITXXNYAN (Seq ID No: 73), complementarity determining region 2 having amino acid sequence XNNYRPP (Seq ID No: 74) and complementarity determining region 3 having amino acid sequence ALWYSNHWV (Seq ID No: 75), interposed between appropriate framework regions, said light chain domain being linked to a heavy chain domain with complementarity determining region 1 having amino acid sequence DYNMY (Seq ID No: 76), complementarity determining region 2 having amino acid sequence YIDPXNGXIFYNQKFXG (Seq ID No. 78) and complementarity determining region 3 having amino acid sequence GGGLFAX (Seq ID No: 78) interposed between appropriate framework regions such that said polypeptide assumes a conformation suitable for degrading cocaine.

In another embodiment, the invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence RSSSGTITANNYGS (Seq ID No. 40), complementarity determining region 2 having amino acid sequence VSNNRGP (Seq ID No: 41), complementarity determining region 3

having amino acid sequence ALWNSNHFV (Seq ID No: 42) interposed between appropriate framework regions, said light chain domain being linked to heavy chain domain with complementarity determining region 1 having amino acid sequence TYYIY (Seq ID No: 67), complementarity determining region 2 having amino acid sequence GMNPGNGVTYFNEKFKN (Seq ID No: 68) and complementarity determining region 3 having amino acid sequence VGNLFAY (Seq ID No: 69) interposed between appropriate framework regions such that the polypeptide assumes a conformation suitable for degrading cocaine.

In another embodiment, the invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence RSSXSLLYXDGKTYLN (Seq ID No: 79), complementarity determining region 2 having amino acid sequence LMSTRXS (Seq ID No: 80) and complementarity determining region 3 having amino acid sequence QXFXXYPFT (Seq ID No: 81) interposed between appropriate framework regions, said light chain domain being linked to a heavy chain domain with complementarity determining region 1 having amino acid sequence SDYAWX (Seq ID No: 82), complementarity determining region 2 having amino acid sequence YIRXXXXTRYNPSLXS (Seq ID No: 83) and complementarity determining region 3 having amino acid sequence XHYYGXXX (Seq ID No: 84) interposed between appropriate framework regions such that the polypeptide assumes a conformation suitable for degrading cocaine.

In another embodiment, the invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence KSSQSLLYSDGKTYLN (Seq ID No: 43), complementarity determining region 2 having amino acid sequence LVSKLDS (Seq ID No: 44) and complementarity determining region 3 having amino acid sequence VQGYTFPLT

(Seq ID No: 45) interposed between appropriate framework regions, said light chain domain being linked to heavy chain domain with complementarity determining region 1 having amino acid sequence DHWMH (Seq ID No: 72), complementarity determining region 2 having amino acid sequence TIDLSDTYTGYNQNFKG (Seq ID No: 71) and complementarity determining region 3 having amino acid sequence RGFDY (Seq ID No: 72) interposed between appropriate framework regions such that the polypeptide assumes a conformation suitable for degrading cocaine.

The invention further provides a humanized catalytic antibody.

The invention further provides a humanized catalytic polypeptide.

The invention provides an isolated nucleic acid molecule encoding the light chain of the antibody. Further, the invention provides an isolated nucleic acid molecule encoding the heavy chain of the antibody.

The invention further provides a nucleic acid molecule encoding a single chain polypeptide.

The present invention further provides a pharmaceutical composition for decreasing the concentration of cocaine in a subject which comprises an amount of the claimed antibody effective to degrade cocaine in the subject's blood and a pharmaceutically acceptable carrier.

The present invention further provides a method of decreasing the concentration of cocaine in a subject which comprises administering to the subject an amount of the claimed antibody effective to degrade cocaine in the subject's blood.

The present invention further provides a pharmaceutical composition for treating cocaine overdose in a subject which comprises an amount of the claimed antibody effective to degrade cocaine in the subject's blood and a pharmaceutical acceptable carrier.

The present invention further provides a method for treating cocaine overdose in a subject which comprises administering to the subject an amount of the claimed antibody effective to degrade cocaine in a subject's blood and reduce cocaine overdose in the subject.

The present invention further provides a pharmaceutical composition for treating cocaine addiction in a subject by diminishing an achievable concentration of cocaine which comprises an amount of the claimed antibody effective to degrade cocaine in the subject and a pharmaceutical acceptable carrier.

The present invention further provides a method for treating cocaine addiction in a subject by diminishing the achievable concentration of cocaine which comprises administering to the subject an amount of the claimed antibody effective to degrade cocaine and thereby diminishing the achievable concentration of cocaine in the subject.

Brief Description of the Figures

Figure 1. Hydrolysis of the benzoyl ester of cocaine. Presumed tetrahydral intermediate formed along the reaction pathway is shown. General structure of a phosphonate monoester analogs of the benzoyl ester: TSA 1, TSA 2, TSA 3. TSA 4.

Figure 2. Synthesis of TSA-1.

Figure 3. Synthesis of TSA-2.

Figure 4. Synthesis of TSA-3.

Figure 5. Plot of log (K_m/K_{TSA1}) versus log (k_{cat}/k_{uncat}) for catalytic antibodies generated by TSA1, 2, and 3. Data represented in this figure are from Tables 1 and 2. Linear relationship by least squares method; r=0.85 excluding Mab 15A10 and 8G4G.

Figure 6. Alignment of Amino acid sequences of Lambda light chains, wherein

9A(lam9)vari indicates the amino acid sequence of the variable domain of the Lambda light chain of the antibody 9A3;

19G(lam5) vari indicates the amino acid sequence of the variable domain of the Lambda light chain of the antibody 19G8;

15A10L Vari indicates amino acid sequence of the variable domain of the Lambda light chain of the antibody 15A10;

G7(lam4) vari indicates the amino acid

sequence of the variable domain of the Lambda light chain of the antibody 8G4G;

Figure 7. Alignment of Amino acid sequences of Kappa light chains, wherein

3B9 K vari indicates the amino acid sequence of the variable domain of the Kappa light chain of the antibody 3B9;

6A12 K vari indicates the amino acid sequence of the variable domain of the Kappa light chain of the antibody 6A12;

12H(L2)k vari indicates the amino acid sequence of the variable domain of the Kappa light chain of the antibody 12H1;

2A k vari indicates the amino acid sequence of the variable domain of the Kappa light chain of the antibody 2A10;

E2(L7) k Vari indicates the amino acid sequence of the variable domain of the Kappa light chain of the antibody 8G4E.

Figure 8. Alignment of Amino acid sequence of Heavy chains, wherein

3B9 vari indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 3B9;

6A12 heavy indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 6A12;

12H H vari	indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 12H1;
2AH-3	indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 2A10;
9(H-3)vari	indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 9A3;
19h6-3 vari	indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 19G8;
15A10 Vari	indicates amino acid sequence of the variable domain of the heavy chain of the antibody 15A10;
E2(H8) Vari	indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 8G4E.
G7(H8) vari	indicates the amino acid sequence of the variable domain of the heavy chain of the antibody 8G4G;

Figure 9. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 15A10.

Figure 10. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 15A10.

Figure 11. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 19G8.

Figure 12. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 19G8.

Figure 13. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 9A3.

Figure 14. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 9A3.

Figure 15. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 8G4G.

Figure 16. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 8G4G.

Figure 17. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 3B9.

Figure 18. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 3B9.

Figure 19. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 6A12.

Figure 20. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 6A12.

Figure 21. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 2A10.

Figure 22. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 2A10.

Figure 24. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 12H1.

Figure 25. Nucleotide sequence of the light chain of the anti-cocaine catalytic antibody 8G4E.

Figure 26. Nucleotide sequence of the heavy chain of the anti-cocaine catalytic antibody 8G4E.

Figure 27. The scFv of 3B9 catalytic monoclonal antibody. H1 indicates the complementarity determining region 1 of the heavy chain of the antibody 3B9;
H2 indicates the complementarity determining region 2 of the heavy chain of the antibody 3B9;
H3 indicates the complementarity determining region 3 of the heavy chain of the antibody 3B9;
L1 indicates the complementarity determining region 1 of the light chain of the antibody 3B9;
L2 indicates the complementarity determining region 2 of the light chain of the antibody 3B9;
L3 indicates the complementarity determining region 3 of the light chain of the antibody 3B9;
FLAG indicates an epitope recognized by a known antibody; 6 x His is capable of binding to the metal Nickle; both of the Flag and 6 x His are useful for purifying the scFv.

Figures 28A and 28B.

(A) Hydrolysis of cocaine at the benzoyl ester and at the methyl ester.

(B) Presumed tetrahedral intermediate of benzoyl ester hydrolysis and corresponding phosphonate monoester analog.

Figure 29. Log dose-response relationship for Mab 15A10 on survival after LD_{90} cocaine. Male rats received intravenous saline (n=8), or Mab 15A10 at 5 mg/kg (n=5), 15 mg/kg (n=5) or 50 mg/kg (n=5) in total volume 5 ml over 5 min. After 5 min, all animals received an intravenous catecholamine infusion as described[18] and an infusion of cocaine (16 mg/kg) at a rate of 1 mg/kg/min. "Survivors" completed the infusion without cardiopulmonary arrest and were observed for one hour after infusion. The effect of Mab 15A10 on survival was significant by X-square test ($p<0.001$).

Figures 30A-30D.

Saturation of Mab 15A10 with cocaine.

(A and B) Mean cocaine dose at seizure **(A)** and at death **(B)**.

(C and D) Plasma concentration of ecgonine methyl ester (EME) **(C)** and cocaine at death **(D)**. To rats prepared as in Figure 2, saline (n=17) or Mab 15A10 100 mg/kg (n=4) or Mab 1C1 100 mg/kg (n=4) in a total volume of 5 ml was administered intravenously over 5 min. Cocaine was infused intravenously at a rate of 1 mg/kg/min until cardiopulmonary arrest. Arterial plasma samples were obtained at death for determination of ecgonine methyl ester and cocaine concentrations. The significance of differences between groups, as described in the text, was determined by Wilcoxon's Rank Sign test with Bonferroni's correction for multiple comparisons.

Detailed Description of the Invention

The invention provides catalytic antibody capable of degrading cocaine characterized by comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is RSSXGTITXXNYAN (Seq ID No: 73), the amino acid sequence of complementarity determining region 2 is XNNYRPP (Seq ID No: 74) and the amino acid sequence of complementarity determining region 3 is ALWYSNHWV (Seq ID No: 75) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is DYNMY (Seq ID No: 76), the amino acid sequence of complementarity determining region 2 is YIDPXNGXXFYNQKFXG (Seq ID No. 78) and the amino acid sequence of complementarity determining region 3 is GGGLFAX (Seq ID No: 78).

The present invention also provides a catalytic antibody capable of degrading cocaine comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is RSSSGTITANNYGS (Seq ID No. 40), the amino acid sequence of complementarity determining region 2 is VSNNRGP (Seq ID No: 41) and the amino acid sequence of complementarity determining region 3 is ALWNSNHFV (Seq ID No: 42) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is TYYIY (Seq ID No: 67), the amino acid sequence of complementarity determining region 2 is GMNPGNGVTYFNEKFKN (Seq ID No: 68) and the amino acid sequence of complementarity determining region 3 is VGNLFAY (Seq ID No: 69).

The present invention also provides a catalytic antibody capable of degrading cocaine comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is RSSXSLLYXDGKTYLN (Seq ID No: 79), the amino acid sequence of Complementarity determining

region 2 is LMSTRXS (Seq ID No: 80) and the amino acid sequence of Complementarity determining region 3 is QXFXXYPFT (Seq ID No: 81) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is SDYAWX (Seq ID No: 82), the amino acid sequence of complementarity determining region 2 is YIRXXXXTRYNPSLXS (Seq ID No: 83) and the amino acid sequence of complementarity determining region 3 is XHYYGXXX (Seq ID No: 84).

The present invention provides a catalytic antibody capable of degrading cocaine comprising a light chain wherein the amino acid sequence of complementarity determining region 1 is KSSQSLLYSDGKTYLN (Seq ID No: 43), the amino acid sequence of complementarity determining region 2 is LVSKLDS (Seq ID No: 44) and the amino acid sequence of Complementarity determining region 3 is VQGYTFPLT (Seq ID No: 45) and a heavy chain wherein the amino acid sequence of complementarity determining region 1 is DHWMH (Seq ID No: 72), the amino acid sequence of complementarity determining region 2 is TIDLSDTYTGYNQNFKG (Seq ID No: 71) and the amino acid sequence of complementarity determining region 3 is RGFDY (Seq ID No: 72).

There are five classes of human antibodies. Each has the same basic structure consisting of two identical polypeptides called heavy chains (molecular weight approximately 50,000 Daltons and two identical light chains, (molecular weight approximately 25,000 Daltons).

Each of the five antibody classes has a similar set of light chains and a distinct set of heavy chains.

A light chain is composed of one variable and one constant domain, while a heavy chain is composed of one variable and three or more constant domains. The combined variable domains of a paired light and heavy chain are known as the Fv region. The Fv determines the specificity of the immunoglobulin, the constant regions have other functions. Amino acid sequence data indicate that each variable domain comprises three hypervariable regions or loops, called complementarity determining regions flanked by four relatively conserved framework regions (24). The hypervariable regions have been assumed to be responsible for the binding specificity of individual antibodies and to account for the diversity of binding of antibodies as a protein class.

In another embodiment, the present invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence RSSXGTITXXNYAN (Seq ID No: 73), complementarity determining region 2 having amino acid sequence XNNYRPP (Seq ID No: 74) and complementarity determining region 3 having amino acid sequence ALWYSNHWV (Seq ID No: 75), interposed between approprioate framework regions, said light chain domain being linked to a heavy chain domain with complementarity determining region 1 having amino acid sequence DYNMY (Seq ID No: 76), complementarity determining region 2 having amino acid sequence YIDPXNGXIFYNQKFXG (Seq ID No. 78) and complementarity determining region 3 having amino acid sequence GGGLFAX (Seq ID No: 78) interposed between appropriate framework regions such that said polypeptide assumes a conformation suitable for degrading cocaine.

In another embodiment, the invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence RSSSGTITANNYGS (Seq ID No. 40), complementarity determining region 2 having amino acid sequence VSNNRGP (Seq ID No: 41), complementarity determining region 3 having amino acid sequence ALWNSNHFV (Seq ID No: 42) interposed between appropriate framework regions, said light chain domain being linked to heavy chain domain with complementarity determining region 1 having amino acid sequence TYYIY (Seq ID No: 67), complementarity determining region 2 having amino acid sequence GMNPGNGVTYFNEKFKN (Seq ID No: 68) and complementarity determining region 3 having amino acid sequence VGNLFAY (Seq ID No: 69) interposed between appropriate framework regions such that the polypeptide assumes a conformation suitable for degrading cocaine.

In another embodiment, the invention provides a polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence RSSXSLLYXDGKTYLN (Seq ID No: 79), complementarity determining region 2 having amino acid sequence LMSTRXS (Seq ID No: 80) and complementarity determining region 3 having amino acid sequence QXFXXYPFT (Seq ID No: 81) interposed between appropriate framework regions, said light chain domain being linked to a heavy chain domain with complementarity determining region 1 having amino acid sequence SDYAWX (Seq ID No: 82), complementarity determining region 2 having amino acid sequence YIRXXXXTRYNPSLXS (Seq ID No: 83) and complementarity determining region 3 having amino acid sequence XHYYGXXX (Seq ID No: 84) interposed between appropriate framework regions such that the polypeptide assumes a conformation suitable for degrading cocaine.

In another embodiment, the invention provides a

polypeptide comprising a light chain domain with complementarity determining region 1 having amino acid sequence KSSQSLLYSDGKTYLN (Seq ID No: 43), complementarity determining region 2 having amino acid sequence LVSKLDS (Seq ID No: 44) and complementarity determining region 3 having amino acid sequence VQGYTFPLT (Seq ID No: 45) interposed between appropriate framework regions, said light chain domain being linked to heavy chain domain with complementarity determining region 1 having amino acid sequence DHWMH (Seq ID No: 72), complementarity determining region 2 having amino acid sequence TIDLSDTYTGYNQNFKG (Seq ID No: 71) and complementarity determining region 3 having amino acid sequence RGFDY (Seq ID No: 72) interposed between appropriate framework regions such that the polypeptide assumes a conformation suitable for degrading cocaine.

The complementarity determining region of the variable domain of each of the heavy and light chains of native immunoglobulin molecules are responsible for antigen recognition and binding.

It has also been discovered that biosynthetic domains mimicking the structure of the two chains of an immunoglobulin binding site may be connected by a polypeptide linker while closely approaching, retaining and often improving their collective binding properties.

The binding site of the polypeptide comprises two domains, one domain comprises variable domain of an immunoglobulin light chain and the other domain comprises variable domain of an immunoglobulin heavy chain. The two domains are linked by a polypeptide. Polypeptides held the two domains in proper conformation to degrade cocaine.

In a preferred embodiment, the invention provides a

hybrid single polypeptide chain comprising variable fragment of a light chain and a variable fragment of an heavy chain, wherein the complementarity determining regions and the framework regions come from separate immunoglobulins.

In another preferred embodiment, the present invention a humanized single chain polypeptide the framework regions are of human or mammalian origin.

The use of mouse non-human antibodies have certain drawbacks particularly in repeated therapeutic regimens. Mouse antibodies, for example, do no fix human complement well, and lack other important immunoglobulin functional characteristics when used in humans. Perhaps, more importantly, antibodies contains stretches of amino acid sequences that will be immunogenic when injected into human patient. Studies have shown that, after injection of a foreign antibody, the immune response elicited by a patient against an antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment.

The present invention thus provides hybrid antibodies such as the "humanized" antibodies (e.g. mouse variable regions joined to human or to other mammalian constant regions) by using recombinant DNA technology, capable of degrading cocaine. The claimed hybrid antibodies have one or more complementarity determining regions from one mammalian source, and framework regions from human or other mammalian source.

The hybrid antibodies of the present invention may be produced readily by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a

first sequence coding for human-like antibody framework regions and a second sequence set coding for the desired antibody complementarity determining regions can be produced synthetically or by combining appropriate DNA and genomic DNA segments.

In order to improve the immunogenicity of the hybrid antibody of the present invention, the human-like immunoglobulin, called acceptor, is selected to have one of the most homologous sequences to the corresponding parts of the immunoglobulin donor. The human-like immunoglobulin framework sequence will typically have about 65% to 70% homology or more to the donor immunoglobulin framework sequences.

The hybrid antibodies will typically comprise at least about 3 amino acids from the donor immunoglobulin addition to the complementarity determining regions. Usually, at least one of the amino acid immediately adjacent to the complementarity determining regions is replaced. Also, the amino acid in the human framework region of an acceptor immunoglobulin is rare for that position and the corresponding amino acid in the donor immunoglobulin is common for that position in human immunoglobulin sequences.

Finally, the amino acid which is predicted to be within about 3 Angstrom of the complementarity determining region in a three-dimensional immunoglobulin model and capable of interacting with the antigen or with the complementarity determining region of the humanized antibody.

When combined into an hybrid antibody, the humanized light and heavy chains or complementarity determining regions and framework regions, of the present invention will be substantially non-immunogenic in humans and

retain the capacity of degrading cocaine as the donor antibody.

The present invention further provides a pharmaceutical composition for decreasing the concentration of cocaine in a subject which comprises an amount of the claimed antibody effective to degrade cocaine in the subject's blood and a pharmaceutically acceptable carrier.

The present invention further provides a method of decreasing the concentration of cocaine in a subject which comprises administering to the subject an amount of the claimed antibody effective to degrade cocaine in the subject's blood.

The present invention further provides a pharmaceutical composition for treating cocaine overdose in a subject which comprises an amount of the claimed antibody effective to degrade cocaine in the subject's blood and a pharmaceutical acceptable carrier.

The present invention further provides a method for treating cocaine overdose in a subject which comprises administering to the subject an amount of the claimed antibody effective to degrade cocaine in a subject's blood and reduce cocaine overdose in the subject.

The present invention further provides a pharmaceutical composition for treating cocaine addiction in a subject by diminishing an achievable concentration of cocaine which comprises an amount of the claimed antibody effective to degrade cocaine in the subject's blood and a pharmaceutical acceptable carrier.

The present invention further provides a method for treating cocaine addiction in a subject by diminishing

the achievable concentration of cocaine which comprises administering to the subject an amount of the claimed antibody effective to degrade cocaine and thereby diminishing the achievable concentration of cocaine in the subject's blood.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

FIRST SERIES OF EXPERIMENTS
General Methods

Unless otherwise noted, reactions were carried out in oven-dried glassware under an atmosphere of argon. Reagent and solvent transfers were made with oven-dried syringes and needles. Dichloromethane, tetrahydrofuran (THF), and benzene were continuously distilled from calcium hydride; a fumehood was used for procedures requiring benzene or chloroform. ^{3}H-phenyl-cocaine was prepared as previously reported (8); radiolabeled materials were handled with appropriate caution. All reagents were purchased from Aldrich Chemical Co. All chromatography solvents were obtained commercially and used as received. Reactions were monitored by analytical thin-layer chromatographic methods (TLC) with the use of E. Merck silica gel 60F glass plates (0.25 mm). Flash chromatography was carried out with the use of E. Merck silica gel-60 (230-400 mesh) as described by Still (29). High-pressure liquid chromatography (HPLC) was performed on a system of Waters 590 using a Dynamax-C_8 (21.4 x 250 mm) column and a detector set at 220 nm. Solvent system was acetonitrile-water (0.1% trifluoroacetic acid).

All carbon NMR spectra were obtained at ambient temperature on either a Bruker AMX-500 (500MHz) spectrometer equipped with a 5 mm broad band inverse probe, Varian VXR-300 (300 MHz) or a Varian Gemini Varian (50 MHz). All proton NMR spectra (400 MHz) were obtained at ambient temperature on a Bruker AM-400 spectrometer, chemical shifts (δ) are reported in parts per million relative to internal tetramethylsilane (0.00 ppm).

FAB high resolution mass spectrometric analysis were performed at Michigan State University, Mass Spectrometry Facility. EI Mass spectrometric analysis were performed at Columbia University, Mass Spectrometry Facility on a JEOL DX303 HF instrument. All results were within 5 ppm of calculated values.

Free TSA 4. Ecgonine methyl ester free base was generated by passing a MeOH solution of ecgonine methyl ester hydrochloride through an Amerlite IRN methoxide-exchange column (Polyscience, Inc). To ecgonine methyl ester (0.049g, 0.25 mmol) in CH_2Cl_2 (10 ml) at 0°C were added phenylphosphonic dichloride (0.042 ml, 0.30 mmol), 1*H*-tetrazole (catalytic) and *N,N*-diisopropylethyl amine (0.11 ml, 3.4 mmol). The reaction was allowed to warn to room temperature. After stirring for 12 h , MeOH (0.150 ml) was added and after 4 h the reaction was concentrated *in vacuo*. Chromatographic purification (SiO_2, $CHCl_3$/MeOH 99:1) afforded the mixed diester 4 (0.042g, 52%) as an oil. To the methyl ester of 4 (0.030g, 0.095 mmol) dissolved in CH_2Cl_2 (3 ml) was added trimethylsilyl bromide (0.05 ml, 0.38 mmol) at room temperature for 2 h. The reaction was concentrated *in vacuo*. Water (5 ml) was added and the reaction was extracted with $CHCl_3$ (5 ml x 2). The organic portions were extracted with another 5 ml of water. The combined aqueous fractions were concentrated *in vacuo*. The residue was taken up in MeOH (5 ml) and propylene oxide (excess) was added. After concentration *in vacuo*, the free TSA 4 (29 mg, 90%) was precipitated as a white solid from a solution of the crude product in $CHCl_3$. ^1H NMR (400 MHz, D_2O) δ 7.51 (m, 2H), 7.32 (m, 3H), 4.37 (m, 1H), 3.83 (m, 1H), 3.67 (m. 1H), 3.54 (s, 3H), 2.95 (m, 1H), 2.54 (s, 3H), 2.14-1.92 (m, 3H), 1.91-1.74 (m, 3H). ^{13}C NMR (300 MHz, D_2O) δ 179.21, 139.31, 136.92, 136.43, 136.30. 134.00, 133.81, 69.24, 69.04, 68.57, 58.45, 53.49, 43.96, 40.17, 28.95, 27.83; high resolution mass spectrum (FAB) for $C_{16}H_{23}NO_5P$

(M+1) calcd 340.1314, found 340.1319.

Compound 5. To ecgonine HCl (0.35 g, 1.6 mmol) in MeOH (4 ml) were added DMF (40 ml), Me_4NOH (2.7 ml, 6.4 mmol), and 1-azido-4-iodobutane (1.8 g, 8 mmol). The reaction was stirred at 50°C for 12 h and then concentrated *in vacuo*. Chromatographic purification (SiO_2, EtOAc/MeOH/NH_4OH 9:0.9:0.1) afforded the ester (0.35 g, 78%) as an oil: ^1H NMR (400 MHz, $CDCl_3$) δ 4.23 (m, 1H), 4.12 (m, 1H), 3.81 (m, 1H), 3.58 (m, 1H), 3.26 (t, 2H, J = 7.0 Hz), 3.18 (m, 1H), 2.74 (t, 1H, J = 4.7 Hz), 2.19 (s, 3H), 2.03 (m, 2H), 1.98-1.63 (m, 6H), 1.61-1.47 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.73, 64.37, 64.29, 63.56, 61.58, 51.74, 50.94, 41.23, 40.26, 25.92, 25.61, 25.51, 24.82; high resolution mass spectrum (FAB) for $C_{13}H_{23}N_4O_3$ (M+1) calcd 283.1770, found 283.1783.

Compound 6. To alcohol 5 (0.43 g, 1.5 mmol) in benzene (10 ml) at 0°C, were added phenylphosphonic dichloride (0.27 ml, 1.7 mmol), 1*H*-tetrazole (8 mg), and *N,N*-diisopropylethyl amine (0.6 ml, 3.4 mmol). The reaction was allowed to warm to room temperature and a precipitate was observed after 15 min. After stirring for 12 h, MeOH (0.1 ml) was added and after 4 h the reaction was concentration *in vacuo*. Chromatographic purification (SiO_2, $CHCl_3$/MeOH/NH_4OH 9.5:0.5:0.02), afforded the mixed diester as a mixture of diastereomers (0.53 g, 89%) as an oil: ^1H NMR (400 MHz, $CDCl_3$) δ 7.73 (m, 2H), 7.60 (m, 1H), 7.49 (m, 2H), 5.09 (m, 1/2H), 4.98 (m, 1/2H), 4.24 (m, 2H), 4.15-3.96 (m, 2H), 3.71 (d, 3/2H, J = 14.6 Hz), 3.68 (d, 2H, J = 14.6 Hz), 3.35-3.15 (m, 3H), 2.91 (s, 3/2H), 2.89 (s, 3/2H), 2.87 (t, 1/2H, J = 7.5 Hz), 2.59 (t, 1/2H, J = 7.5 Hz), 2.43-2.22 (m, 5/2H), 2.17-1.95 (m, 5/2H), 1.71-1.57 (m, 2H), 1.39 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 161.55, 149.12, 134.32, 132.55, 129.80, 129.66, 66.72, 66.54, 66.45, 66.28, 64.80, 63.90, 63.81, 53.81, 51.60, 51.50, 49.58, 49.15, 40.30, 35.60, 35.27,

26.35, 26,06, 26.02, 25.82, 25.10, 23.98; high resolution mass spectrum (FAB) for $C_{20}H_{30}N_4O_5$ (M+1) calcd 437.1954, found 437.1953.

Compound 7. Me_3P (1.1 ml, 1M in THF, 1.1 mmol) was added to azide 6 (0.217 g, 0.5 mmol) in 6 ml THF/MeOH/H_2O (9:9:2) and the reaction was stirred at room temperature for 5 h. After concentration *in vacuo*, the crude unstable amine (36 mg, 0.084 mmol) was taken up in dry CH_2Cl_2 (5 ml) and 1, 4-^{14}C-succinic anhydride (9 mg, 0.093 mmol) was added. The reaction was stirred under Ar for 12 h and then concentrated. For purification, the crude acid 7 (44 mg, 0.087 mmol) was esterified in CH_2Cl_2 (10 ml) with DCC (36 mg, 0.17 mmol), benzyl alcohol (36 μl, 0.35 mmol), and DMAP (cat). The reaction was stirred for 12 h and concentrated. Chromatographic purification (SiO_2, 0.5:99.5 MeOH/$CHCl_3$ and 2:98 MeOH/$CHCl_3$) afforded the benzyl ester of 7 as a mixture of diastereomers (32 mg, 59%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (m, 2H), 7.62 (m, 1H), 7.49 (m, 2H), 7.33 (m, 5H), 6.64 (br. s, 1/2H), 6.56 (br. s, 1/2H), 5.10 (s, 2H), 4.96 (m, 1/2H), 4.89 (m, 1/2H), 4.38-3.85 (m, 4H), 3.74 (d, 3/2H, J = 15.2 Hz), 3.68 (d, 3/2H, J = 15.2 Hz), 3.32-3.12 (m, 3H), 2.89 (s, 3/2H), 2.87 (s, 3/2H), 2.70-2.59 (m, 3H), 2.52-2.26 (m, 4H), 2.10-1.97 (m, 2H), 1.68 (m, 1H), 1.55 (m, 1H), 1.38 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.55, 172.66, 171.37, 161.62, 161.28, 136.59, 134.17, 132.37, 129.56, 129.24, 128.88, 128.71, 67.04, 66.81, 66.64, 66.25, 64.66, 63.75, 53.74, 49.37, 49.00, 40.11, 39.42, 35.55, 35.26, 31.35, 30.31, 26.19, 26.06, 24.89, 23.91; high resolution mass spectrum (FAB) for $C_{31}H_{42}N_2O_8P$ (M+1) calcd 601.2679, found 601.2682.

The benzyl ester of 7 (17 mg, 0.028 mmol) in methanol (10 ml) was stirred with a catalytic amount of Pd on C (10%) under H_2 (1 atm) for 4 h. The reaction mixture was

filtered and concentrated *in vacuo* to provide acid 7 quantitatively. ^{1}H NMR (400 MHz, CD_3OD) δ 7.69 (m, 2H), 7.60 (m, 1H, 7.51 (m, 2H), 4.99 (m, 1H), 4.20-4.08 (m, 2H), 3.89 (m, 1H), 3.73 (d, 3/2H, J = 21.5 Hz), 3.66 (d, 3/2H, J=21.5 Hz), 3.62 (m, 1H), 3.22 (m, 1H), 3.10 (m, 1H), 3.01 (m, 1H), 2.76 (s, 3/2H), 2.75 (s, 3/2H), 2.50 (m, 2H), 2.38-2.28 (m, 5H), 2.04 (m, 2H), 1.61 (m, 1H), 1.50 (m, 1H), 1.34 (m, 3H); ^{13}C NMR (500 MHz, CD_3OD) δ 176.22, 174.52, 173.47, 162,22, 134.97, 132.79, 130.18, 67.66, 67.53, 66.99, 65.47, 64.44, 53.89, 39.63, 39.33, 35.99, 31.50, 30.23, 26.71, 24.65, 23.67; high resolution mass spectrum (EI) for $C_{24}H_{36}N_2O_8P$ calcd 511.2209 (M+1), found 511.2218.

Compound 8. To the acid 7 (40mg, 0.078 mmol) dissolved in acetonitrile (5ml) was added *N*-hydroxyphthalimide (14 mg, 0.086 mmol) and DCC (32 mg, 0.16 mmol). After 1 h at room temperature a white precipitate formed. The reaction was concentrated *in vacuo*. The crude activated ester was taken up in CH_2Cl_2 (5 ml) and trimethylsilyl bromide (100 μl, 0.78 mmol) was added. The reaction was stirred for 1 h and concentrated *in vacuo*. The crude reaction mixture was taken up in acetonitrile (5ml) and amylamine (100 μl, 0.78 mmol) was added. A bright orange color developed immediately and faded to light yellow in 1 h. Another portion of amylamine (100 μl) was added. The reaction was stirred for 12 h at room temperature and concentrated *in vacuo*. Water (3 ml) was added and the reaction was extracted with $CHCl_3$ (5ml x 2). The organic portions were extracted with another 5 ml of water. The combined aqueous fractions were concentrated *in vacuo*. High pressure liquid chromatography on a Dynamax 300 Å, 12 μ, C-8 (10 x 250 mm) column eluting with 4%-40% CH_3CN/H_2O gradient (0.1% trifluoroacetic acid) provided the amide 8 (16 mg, 36% yield). ^{1}H NMR(400 MHz, CD_3OD) δ 7.72(m, 2H), 7.56(m, 1H), 7.47(m, 2H), 4.12(m, 3H), 3.87(m, 1H), 3.23(m, 2H), 3.14(m, 3H), 2.77(m, 4H),

2.58(m, 4H), 2.34(m, 3H), 2.16(m, 1H), 1.97(m, 2H), 1.55-1.48(m, 6H), 1.26(m, 4H), 0.846(t, 3H, J = 6.3 Hz); ^{13}C NMR (500 MHz, CD_3OD) δ 175.76, 173.62, 133.83, 132.23, 131.01, 129.07, 66.56, 66.52, 65.26, 64.33, 41.13, 40.36, 39.33, 35.93, 31.13, 29.91, 29.48, 28.95, 26.57, 26.28, 24.73, 23.66, 23.22; high resolution mass spectrum (FAB) for $C_{28}H_{45}N_3O_7P$ calcd 566.2995 (M+1), found 566.2997.

TSA 1. Acid 7 (14 mg, 0.027 mmol) in CH_3CN (5 ml), was stirred at room temperature with *N*-hydroxyphthalimide (4.8 mg, 0.029 mmol) and DCC (11 mg, 0.053 mmol). A red color developed immediately. After 2.5 h, the reaction was partially concentrated *in vacuo*, filtered through a small cotton plug and then fully concentrated. The crude, unstable activated ester (0.027 mmol assumed) was taken up in CH_2Cl_2 (5 ml) and trimethylsilyl bromide (20 μl, 0.15 mmol) was added. The reaction was stirred for 1 h and concentrated *in vacuo*. BSA (5 mg) or ovalbumin (5 mg) in $NaHCO_3$ (5 ml, 1 N, pH 8.0) at 0°C was added and the mixture vigorously stirred. The reaction was allowed to warm to room temperature and, after 1 h, terminated by gel filtration chromatography (Sephadex G-25 M, pH 7.4 PBS). Protein-containing fractions were combined and dialyzed against PBS at 4°C overnight (pH = 7.4, 3 x 1000 ml). The coupling efficiency was estimated to be 6:1 for BSA and 15:1 for ovalbumin based on incorporation of radiolabel.

Compound 9a. To 2-(p-bromophenyl)ethanol (1.3 g, 6.5 mmol) were added methylene chloride (20 ml), *t*-butyldimethylsilyl chloride (1.07 g, 7.1 mmol) and imidazole (660 mg, 9.7 mmol). The reaction was stirred at room temperature for 12 h, filtered and concentrated *in vacuo*. Chromatographic purification (SiO_2 95:5 hexane: $CHCl_3$) afforded the silyl ether (1.28 g, 66%). To the ether (792 mg, 2.51 mmol) in THF (25 ml) under Ar at -78°C

was added *n*-BuLi (1.2 ml, 2.3 M hexanes, 2.76 mmol) dropwise. The reaction was stirred for 30 min and a solution of diethylchlorophosphate (370 μl, 2.5 M THF, 0.93 mmol) was added. The reaction was stirred at -78°C for an additional 5 min and allowed to warm to room temperature. Aqueous NH_4Cl (20 ml) was added and the reaction was extracted with EtOAc (3x10 ml). The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. THF (10 ml) and aq Bu_4NF (2.5 ml, 1 M, 2.5 mmol) were added to the residue. This solution was stirred at room temperature for 30 min and concentrated *in vacuo*. Chromatographic purification (SiO_2, 9:1 EtOAc/MeOH), provided the alcohol 9a (229 mg, 35%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, 2H, J = 12.5, 7.1 Hz), 7.33 (dd, 2H, J = 12.5, 4.5 Hz), 4.11 (m, 4H), 2.92 (t, 2H, J = 6.5 Hz), 2.89 (t, 2H, J = 6.5 Hz), 1.32 (t, 6H, J = 7.8 Hz). ^{13}C NMR (50 MHz, $CDCL_3$) δ 144.32, 132.51, 129.78, 129.47, 63.61, 62.69, 39.74, 16.98; high resolution mass spectrum (El) for $C_{12}H_{20}O_4P$ calcd 259.1099 (M+1), found 259.1092.

Compound 9b. To alcohol 9a (193 mg, 0.75 mmol) were added CH_2Cl_2 (7.5 ml), Et_3N (115 μl, 0.83 mmol), TsCl (145 mg, 0.75 mmol), DMAP (catalytic). The reaction was stirred at room temperature for 12 h. Concentration and purification (SiO_2, 3:1 EtOAc:hexane) provided the tosylate (251 mg, 81.5%) and to a portion of this product (232 mg, 0.56 mmol) were added benzene (3 ml), water (3 ml), tricaprylmethyl ammonium chloride (cat.), and NaN_3 (150 mg, 2.25 mmol). The reaction was refluxed at 65°C for 12 h. Saturated aq NH_4Cl (5 ml) was added, and the reaction was extracted with EtOAc. The combined organic layers were treated with $MgSO_4$, filtered, and dried *in vacuo*. Chromatography (SiO_2, 1:1 hexane:EtOAc) afforded the azide 9b (137 mg, 86%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, 2H, J = 12.5, 7.1 Hz), 7.32 (dd, 2H, J = 12.5, 4.5 Hz), 4.09 (m, 4H), 3.86 (t, 2H, J = 7.5 Hz), 2.92

(t, 2H, J = 7.5 Hz), 1.32 (t, 6H, J = 7.3 Hz). ^{13}C NMR (50 MHz, $CDCl_3$) δ 143.31, 132.65, 129.50, 129.20, 125.31, 62.58, 52.47, 35.89, 16.94; high resolution mass spectrum (EI) for $C_{12}H_{19}N_3O_2$ calcd 284.1164 (M+1), found 284.1168.

Compound 10. Diethyl phosphonate ester 8b (600 mg, 2.12 mmol) in CH_2Cl_2 (5 ml) were stirred with trimethylsilyl bromide (1 ml, 11 mmol) and warmed to 45°C. After 20 min, it was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (3.2 ml), oxalyl chloride (3.2 ml, 2M in CH_2Cl_2, 6.36 mmol) and one drop of DMF were added. After stirring 20 min at room temperature, the volatiles was removed *in vacuo*. The unstable phosphonic dichloride was used directly.

Compound 11. Ecgonine methyl ester free base was generated as described for compound 4. To ecgonine methyl ester (170 mg, 0.854 mmol) in benzene (20 ml) at 0°C was added *N,N*-diisopropylethylamine (0.74 ml, 4.26 mmol), 1*H*-tetrazole (catalytic) and the phosphonic dichloride 10 (225 mg, 0.854 mmol). The reaction was allowed to warm to room temperature and stirred for 12 h. Methanol (3 ml) was added and after 20 min the reaction mixture was concentrated *in vacuo*. Chromatographic purification (SiO_2, 1:9 MeOH:$CHCl_3$) afforded the mixed diester as a mixture of diastereomers (108 mg, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (m, 2H), 7.29 (m, 2H), 4.63 (m, 1H), 3.73 (s, 3/2H), 3.70 (s, 3/2H), 3.63 (d, 3/2H, J = 11.4 Hz), 3.62 (d, 3/2H, J = 11.4 Hz), 3.51 (t, 2H, J = 7.2 Hz), 3.48-3.39 (m, 1H), 3.23-3.15 (m, 1H), 3.05 (m, 1/2H), 2.91 (t, 2H, J = 7.2 Hz), 2.75 (m, 1/2H), 2.57-2.26 (m, 1H), 2.14 (s, 3H), 2.09-1.52 (m, 5H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.91, 170.65, 143.27,132.80,132.61, 129.45, 129.11, 125.08, 78.22, 77.73, 76.95, 70.15, 65.31, 62.14, 52.50, 52.84, 52.15, 41.56, 37.84, 35.97, 25.70, 25.58; high resolution mass spectrum (EI) for $C_{19}H_{27}N_4O_5P$ calcd 422.1719 (M^+), found

Compound 12. To azide 11 (370 mg, 0.877 mmol) was added THF (9 ml) and triphenylphosphine (400 mg, 1.75 mmol). After stirring at r.t. for 12 h, water (1 ml) was added. The mixture was stirred for 3 h and concentrated *in vacuo*. To the crude amine (200 mg, 0.51 mmol) were added CH_2Cl_2 (7.5 ml) and succinic anhydride (3.5 mg, 0.35 mmol). The reaction was stirred for 12 h and concentrated *in vacuo*. The crude acid 12 (290 mg, 0.51 mmol) was dissolved in CH_2Cl_2 (10 ml) and DCC (200 mg, 0.97 mmol), DMAP (catalytic) and benzyl alcohol (0.2 ml, 1.9 mmol) were added. The reaction was stirred at room temperature for 12 h and concentrated *in vacuo*. Chromatography SiO_2, 10:10:0.4 $CHCl_3$: EtOAc: NH QH) afforded the benzyl ester of 12 (197 mg, 65%) as a mixture of diastereomers. ^1H NMR (400 MHz, $CDCl_3$) δ 7.79-7.61 (m, 4H), 7.33-7.25 (m, 5H), 5.11 (s, 2H), 4.69-4.58 (m, 1H), 3.73 (s, 3/2H), 3.69 (d, 3/2H, J = 18.1 Hz), 3.62 (d, 3/2H, J = 18.1 Hz), 3.59 (s, 3/2H), 3.46 (m, 2H), 3.27-3.03 (m, 3H), 2.81 (t, 2H, J = 7.2 Hz), 2.69 (t, 2H, J = 6.8 Hz), 2.42 (t, 2H, J = 6.8 Hz), 2.15 (s, 3H), 2.08-1.80 (m, 3H), 1.69-1.51 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.35, 171.42, 132.38, 132.11, 129.99, 129.93, 129.80, 129.67, 129.61, 129.56, 129.48, 129.94, 128.66, 128.49, 67.07, 66.16, 66.43, 63.40, 53.28, 50.49, 50.18, 50.06, 49.64, 49.36, 49.21, 48.79, 39.58, 36.14, 31.14, 30.07, 24.73; high resolution mass spectrum (EI) for $C_{30}H_{39}N_2O_8P$ calcd 586.2444 (M^+), found 586.2428.

Acid 12 was quantitatively regenerated from the benzyl ester as described for acid 7 as a mixture of diastereomers. ^1H NMR(400 MHz, $CDCl_3$) δ 7.74 (m, 2H), 7.60 (m, 1H), 7.49 (m, 2H), 5.02 (m, 1/2H), 4.92 (m, 1/2H), 4.24 (m, 2H), 3.83 (s, 3/2H), 3.74 (d, 3/2H, J = 12 Hz), 3.67 (d, 3/2H, J = 12 Hz), 3.51 (s, 3/2H), 2.79 (m, 1H), 2.75 (s, 3/2H), 2.74 (s, 3/2H), 2.45 (m, 1H),

2.35 (m, 6H), 2.02 (m, 2H), 1.20 (m, 4H); ^{13}C NMR (300 MHz, CD_3 OD) δ 175.92, 174.33, 173.72, 147.06, 132.85, 132.72, 130.62, 130.41, 129.56, 129.29, 67.31, 65.28, 64.37, 53.69, 53.43, 53.24, 41.25, 39.21, 36.42, 35.83, 35.70, 31.35, 30.58, 30.07, 24.52, 23.50; high resolution mass spectrum (EI) for $C_{23}H_{34}N_2O_8P$ calcd 497.2053 (M+1), found 497.2064.

Compound 13. To the acid 12 (23 mg, 0.049 mmol) dissolved in acetonitrile (5 ml) was added *N*-hydroxyphthalimide (9 mg, 0.054 mmol) and DCC (20 mg, 0.097 mmol). Reaction with trimethylsilyl bromide (0.65 ml, 0.49 mmol) and amylamine (0.57 ml, 0.47 mmol) proceeded by the protocols developed for compound 8 to yield amide 13 (8 mg, 30% yield). ^1H NMR: (400 MHz, CD_3OD) 7.69 (m, 2H), 7.32 (m, 2H), 4.75 (m, 1H), 4.08 (m, 1H), 3.86 (m, 1H), 3.71 (s, 3H), 3.39 (m, 3H), 3.14 (m, 2H), 2.82 (m, 5H), 2.42 (s, 3H), 2.38-2.22 (m, 4H), 2.13-2.00 (m, 3H), 1.49 (m, 2H), 1.32 (m, 4H), 0.91 (t, 3H, J=1.5Hz) ^{13}C NMR (500 MHz, CD_3OD) δ 173.39, 159.53, 159.22, 144.10, 132.23, 130.95, 129.61, 117.04, 64.83, 64.62, 64.12, 63.92, 62.53, 40.89, 39.54, 36.83, 36.23, 34.31, 31.21, 30.52, 30.14, 29.24, 27.94, 23.95, 21.47; high resolution mass spectrum EI for $C_{27}H_{43}N_3O_7P$ calcd 552.2839 (M+1), found 552.2863.

TSA 2. To acid 12 (70 mg, 0.14 mmol) were added DMF (4 ml), DCC (116 mg, 0.57 mmol), and *N*-hydroxyphthalimide (92 mg, 0.57 mmol) at r.t. The reaction was stirred for 12 h at 4°C, concentrated *in vacuo* and filtered through a small cotton plug rinsing with $CHCl_3$ (10 ml). To an aliquot of this solution (2 ml) was added bromotrimethylsilane (0.1 ml, 0.76 mmol). Work-up and coupling proceeded by the protocol developed for TSA 1. The coupling efficiency to BSA was 15 to 1; to ovalbumin 10 to 1.

Compound 14. To *N*-norcocaine (206 mg, 0.713 mmol) and *N,N*-diisopropyethylamine (186 μl, 1.07 mmol) in THF (30 ml) was added 1-azido-4-iodobutane (160 mg, 0.713 mmol) at r.t. The reaction mixture was heated to 60°C for 2 days. Concentration *in vacuo* and chromatographic purification (SiO_2 1:9 EtOAc hexane) yielded the ecgonine ester 14 (205 mg, 75%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, 2H, *J* = 6.0 Hz), 7.58 (t, 1H, *J* = 6.1 Hz), 7.41 (t, 2H, *J* = 7.0 Hz), 5.25 (m, 1H), 3.70 (s, 3H), 3.68 (m, 1H), 3.50 (m, 1H), 3.28 (t, 2H, *J* = 7.4 Hz), 3.03 (m, 2H), 2.43 (m, 1H), 2.26 (m, 2H), 2.04-2.00 (m, 2H), 1.86 (m, 1H), 1.73-1.65 (m, 4H), 1.47 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 171.47, 166.96, 133.77, 131.24, 130.59, 129.16, 68.10, 63.55, 61.24, 52.89, 52.21, 52.05, 53.13, 36.49, 27.29, 26.95, 26.86, 26.34; high resolution mass spectrum (FAB) for $C_{20}H_{27}N_4O_4$ (M+1) calcd 387.2032, found 387.2041.

Compound 15. *N*-substituted cocaine 14 (205 mg, 0.53 mmol) was hydrolyzed with aq HCl (10 ml, 0.7 N) at 90°C for 4 h. The mixture was extracted with ether, concentrated and dissolved in MeOH (25 ml) saturated with HCl(g). After 2 h at 60°, solvent was removed under vacuum, and the residue was dissolved in MeOH and passed through an Amberlite IRN methoxide-exchange column (Polysciences, Inc) (1 ml) to generate the crude free base. Chromatographic purification (SiO_2 5:95 MeOH:$CHCl_3$) afforded alcohol 15 (102 mg, 72%). ^1H NMR (400 MHz, $CDCl_3$) δ 3.80 (m, 1H), 3.69 (s, 3H), 3.03 (m, 1H), 3.66 (m, 2H), 3.24 (t, 2H, *J* = 7.2 Hz), 3.18 (m, 1H), 2.75 (t, 1H, *J* = 5.1 Hz), 2.21 (m, 1H), 1.95-1.78 (m, 4H), 1.61-1.38 (m, 6H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 169.58, 65.55, 62.89, 61.27, 53.10, 52.61, 52.26, 52.18, 41.20, 27.36, 27.08, 27.02, 25.83; high resolution mass spectrum (FAB) for $C_{13}H_{23}N_4O_3$ (M+1) calcd 283.1770, found 283.1779.

Compound 16. To the ecgonine derivative 15 (102 mg, 0.37

mmol) in benzene (15 ml) at 0°C were added 1*H*-tetrazole(catalytic), *N,N*-diisopropylethyl amine (0.163 ml, 0.94 mmol) and phenylphosphonic dichloride (0.67 ml, 0.47 mmol). The reaction mixture was allowed to warm to room temperature overnight. Excess MeOH was added and the mixture was stirred at room temperature for 3 h. Chromatographic purification (SiO_2 5:95 of 4% NH_4OH in MeOH and a 1:1 mixture of hexane and $CHCl_3$) and prep-TLC (2.5:97.5 MeOH: CH_2Cl_2) afforded the mixed diester 16 as a mixture of diastereomers (78 mg, 49%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.66 (m, 2H), 7.62 (m, 1H), 7.49 (m, 2H), 5.08 (m, 1/2H), 4.97 (m, 1/2H), 4.32 (m, 1H), 4.18 (m, 1H), 3.88 (s, 3/2H), 3.75 (d, 3/2H, J = 16.4 Hz), 3.71 (d, 3/2H, J = 16.4 Hz), 3.49 (s, 3/2H), 3.45-3.25 (m, 4H), 2.98 (m, 1H), 2.63-2.22 (m, 4H), 2.19-2.01 (m, 2H), 1.92-1.63 (m, 4H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 160.10, 159.72, 133.37, 133.23, 131.61, 131.53, 131.46, 130.29, 128.86, 128.76, 128.64, 66.76, 63.74, 63.58, 62.55, 62.43, 54.46, 54.17, 52.64, 51.67, 49.11, 48.79, 36.57, 36.28, 26.91, 25.58, 25.18, 24.18; high resolution mass spectrum (FAB) for $C_{20}H_{30}N_4O_5P$ (M+1) calcd 437.1954, found 437.1928.

Compound 17. Me_3P (0.156 ml, 1 M, in THF, 0.157 mmol) was added to azide 16 (12 mg, 0.026 mmol) in MeOH (5 ml) and the reaction was stirred at room temperature for 2 h. After concentration *in vacuo*, the crude amine was taken up in CH_2Cl_2 (5 ml), succinic anhydride (2.6 mg, 0.026 mmol) was added. The reaction mixture was stirred at room temperature overnight and concentrated. The crude acid 17 was dissolved in CH_2Cl_2 (10 ml) and benzyl alcohol (0.05 ml, 0.048 mmol), DCC (10 mg, 0.048 mmol), and DMAP (catalytic) was added. The reaction was stirred overnight at r.t. and concentrated. Column chromatography (SiO_2, 5:95 MeOH:CH_2Cl_2) and prep-TLC (5:95 MeOH CH_2Cl_2) afforded the benzyl ester as a mixture of diastereomers (11 mg, 70% from 13). ^1H NMR (400 MHz, $CDCl_3$) δ 7.76 (m, 2H), 7.63 (m, 1H), 7.51 (m, 2H), 7.32

(m, 5H), 7.01 (br s, 1H), 5.09 (s, 2H), 5.03 (m, 1/2H), 4.94 (m, 1/2H), 4.29-4.09 (m, 2H), 3.83 (s, 3/2H), 3.77 (d, 3/2H, J = 17.1 Hz), 3.69 (d, 3/2H, J = 17.1 Hz), 3.49 (s, 3/2H), 3.38-3.22 (m, 4H), 3.01 (m, 2H), 2.69-2.33 (m, 8H), 2.04-1.60 (m, 6H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 172.94, 172.68, 172.09, 135.86, 133.30, 131.64, 128.90, 128.78, 128.65, 128.54, 128.17, 128.82, 66.24, 65.81, 62.71, 62.54, 61.16, 61.03, 52.95, 51.49, 47.69, 37.64, 35.18, 30.41, 29.39, 25.67, 24.00, 23.54, 21.95; high resolution mass spectrum (FAB) for $C_{31}H_{42}N_2O_8P$ (M+1) calcd 601.2679, found 601.2676.

Acid 17 was quantitatively regenerated from the benzyl ester as described for acid 7. ^{1}H NMR(400 MHz, $CDCl_3$) δ 7.74 (m, 2H), 7.60 (m, 1H), 7.48 (m, 2H), 5.02 (m, 1/2H), 4.92 (m, 1/2H), 4.33-4.09 (m, 2H), 3.83 (s, 3/2H), 3.74 (d, 3/2H, J = 23 Hz), 3.67 (d, 3/2H, J = 23 Hz), 3.51(s, 3/2H), 3.33-3.19 (m, 6H), 2.98 (m, 1H), 2.63 (m, 2H), 2.49 (m, 4H), 2.34 (m, 2H), 2.06-1.96 (m, 2H), 1.81-1.76 (m, 2H), 1.57 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 175.23, 173.41, 172.06, 133.21, 131.65, 128.90, 128.58, 65.87, 62.75, 60.89, 53.30, 52.98, 51.54, 48.16, 47.75, 37.61, 31.02, 30.33, 25.76, 24.15, 23.54, 21.92; high resolution mass spectrum (EI) for $C_{28}H_{36}N_2O_8P$ calcd 511.2209 (M+1), found 511.2213.

Compound 18. To acid 17 (6 mg, 0.012 mmol) dissolved in CH_3CN (3 ml) was added *N*-hydroxyphthalimide (2.2 mg, 0.013 mmol) and DCC (5 mg, 0.024 mmol). Reaction with trimethylsilyl bromide (0.016 ml, 0.12 mmol) and the amylamine (0.14 ml, 0.012 mmol) proceeded by the protocols developed for compound 8 to yield amide 4 (4.4 mg, 65%). ^{1}H NMR: (400 MHz, CD_3OD) δ 7.81 (m, 2H), 7.56-7.38 (m, 3H), 5.95 (m, 1H), 5.39 (m, 1H), 5.05 (m, 1H), 4.79 (s, 3H), 4.29-4.12 (m, 6H), 3.61-3.04 (m, 10H), 2.83-2.34 (m, 11H), 0.94 (t, 3H, J=7.2 Hz). ^{13}C NMR (300 MHz). δ 175.12, 174.98, 174.39, 132.49, 129.36, 129.21,

65.79, 64.72, 62.26, 53.33, 52.52, 40.44, 39.01, 36.78, 32.17, 31.91, 30.23, 30.14, 27.39, 24.69, 24.32, 23.45, 23.22, 14.36; high resolution mass spectrum (FAB) for $C_{28}H_{45}N_3O_7P$ (M+1) calcd 566.2995, found 566.2997.

TSA 3. To the acid 17 (12 mg, 0.023 mmol) and *N*-hydroxyphthalimide (16 mg, 0.096 mmol) in DMF (2 ml) was added DCC (19 mg, 0.096 mmol). The reaction was stirred at 4°C overnight, concentrated *in vacuo*, and filtered with $CHCl_3$ (10 ml) The activated ester was kept as a $CHCl_3$ solution (10 ml) at -20°C and used without purification. Trimethylsilyl bromide (0.050 ml, 0.379 mmol) was added to a 5 ml aliquot of the activated ester at room temperature. Work-up and coupling proceeded by the protocol developed for TSA 1. The coupling ratio to BSA was 11:1; to ovalbumin 12:1

Hybridoma generation

As previously described (9), BALB/c mice were immunized with the analog-carriers and the immune response was followed by ELISA. Hybridomas were prepared by standard methods (9,17).

Hybridoma cells (~ $2x10^6$) were placed either into a mouse peritoneum that had been pretreated with pristane or into T-150 flask cell culture. The harvested ascites or cell culture super natents were subjected to affinity chromatography on a preparative protein A HPLC column (Bio-Rad) (purity > 90% by SDS-polyacrylamide gel electrophoresis). Samples of catalytically active antibodies were purified by anion exchange HPLC with an analytic DEAE column (TOSOH HASS TSK-gel) using 0.02 M Tris and a linear gradient pH 8.8/0.0 M NaCl to pH 7.0/0.3 M NaCl without loss of cocaine esterase activity.

Protocol for binding studies (CIEIA)

Plates were coated with the TSA (tethered to ovalbumin) that elicited the catalytic antibody intended for CIEIA. Free TSA 4 or the TSA-related amides 8, 13, or 14, were tested for inhibition of antibody binding to the eliciting TSA by published protocols (20b).

Protocol for kinetic measurements

Catalytic antibody in 50 mM phosphate-buffered saline pH 8.0 (except 2A10 and 6A12 at pH 7.0) was incubated with ^{3}H-cocaine typically at five concentrations. At three time intervals, aliquots were acidified with cold HCl (aqueous) to a final pH of 2 and partitioned with hexane-diethyl ether (1:1), and the organic phase was assayed by scintillation counting. Background hydrolysis was determined in otherwise identical reactions without antibody, and observed rates were corrected. Assays were performed in triplicate with standard error <10%. As a control, the release of benzoic acid was confirmed by HPLC (Perkin-Elmer) using an analytical reverse-phase C_{18} column (VYDAC) with an acetonitrile-water (0.1% trifluoroacetic acid) gradient and the detector set at 220 nm.

HPLC analysis of a reaction mixture without antibody showed that the methyl ester of cocaine spontaneously hydrolyzes to benzoyl ecgonine with a $t_{1/2}$ = 20 hours (pH 7). Thus, benzoyl ecognine is not available as a benzoyl esterase substrate at the early reaction times of the ^{3}H-cocaine hydrolysis assay and the release of benzoic acid is attributed solely to cocaine hydrolysis.

Amino acid sequencing

Light and heavy chains were separated by SDS-

polyacrylamide gel electrophoresis and then electroblotted to a polyvinylidenedifluoride membrane (30) for direct NH_2-terminal sequencing by automated Edman degradation on an Applied Biosystems 470A or 477A sequencer. To obtain internal sequence, separated bands from 2A10, 19G7, 9A3 and 15A10 were reduced with dithiothreitol, alkylated with iodoacetamide, and cleaved with trypsin (31) in 1M urea, 0.05 M NH_4HCO_3, pH 8.0. The peptide fragments were extracted from the membrane, separated by HPLC (Hewlett-Packard) on a reverse-phase C4 column (VYDAC) using an acetonitrile-water (0.07% trifluoroacetic acid) gradient and sequenced.

Pcr cloning of variable domains

Mouse hybridoma cell lines producing catalytic antibodies were grown to 1x 10^8 cells and total RNA was prepared using a microadaptation of the guanidine thiocyanate/ phenol procedure (32) and selection on a oligo (dT) cellulose column.

Degenerate and non-degenerate oligonucleotide PCR primers were designed using amino acid sequences (2A10,15A10) or the data base of Kabat et al. (24). Restriction endonuclease sites were incorporated into the primers at their 5' prime end to facilitate cloning. The restriction sites utilized were Eco RI, Spe I, Xba I, or Xho I. The sense and antisense oligonucleotide primers for light chain (LC) and heavy chain (HC) of each hybridoma line were as follows: For 9A3,19G8,15A10,8G4E and 8G4G LC: 5'-GGAATTCCACIA/TC/GICCIGGIGAA/GACIG-3' and 5'GCTCGAGCC/TTCA/GTGIGTIACITGA/GCA-3'. For 3B9,6A12 and 12H1 LC:
5'-CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA-3' and
5'-GCGCCGTCTAGAATTAACACTCA TTCCTGT TGAA-3'. For 2A10 LC:
5'-GCTCTAGAGCGAT/CATIGTIATGACICAA/GGAT/CGA-3' and
5'-GGAATTCCA/GTTA/GTGICT/CT/CTCA/GTAT/CTCA/GTC-3'. For

3B9, 6A12, 12H1, 9A3, 19G8, 8G4E and 84G4G HC: 5'-AGGTCCAGCTGCTCGAGTCTGG-3' and 5'-AGGCTTACTAGTACAATCCCTGGGCACAAT-3'. For 2A10 HC: 5'-TCCCAGGTCCAACTGCAGCAGCC-3' and 5'-ATAACCCTTGACCAGGCATCC-3'. For 15A10 HC: 5'-CCAGTTCCGAGCTCGTGATGACACAGTCTCC-3' and 5'-AGCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3'.

DNA templates were synthesized using 0.5 μg of hybridoma mRNA and Moloney murine leukemia virus reverse transcriptase. Amplifications were carried out in a Perkin-Elmer/Cads thermal cycler for 30 cycles of denaturation (96°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 3 min). The PCR products were purified by electrophoresis in 1.5% agarose gel. Isolated PCR products from each reaction were subcloned into Bluescript plasmid and analyzed by DNA sequence analysis for the presence of open reading frame. Nucleotide sequences were assembled using the IBI MacVector 3.0 program.

EXPERIMENTAL RESULTS

Synthesis of transition-state analogs

Phosphonate monoesters, which stably mimic the geometry and charge distribution of the transition-state for 2nd-order ester hydrolysis by hydroxide, have yielded, in some instances, catalytic antibodies of high activity (8). However, such analogs are also known to idiosyncratically fail to elicit any catalytic antibodies and so the rules for analog construction must be defined empirically (11). Strategies to improve analog efficiency have been devised, including "bait and switch" (11) and substrate attenuation (12), but the cost of such expedients is a divergence between analog and substrate structure which results on average in catalytic antibodies with higher values for K_m. Inhalation of vaporized cocaine yields a peak pulmonary vein

concentration (13) of 10-30 μM and this is less than the K_m of most catalytic antibodies with esterase activity. At a sub-saturating concentration of cocaine, a higher K_m would result in a lower turnover rate and increase the already limiting requirement for a high k_{cat}. Thus, the construction of a high fidelity analog that differed from cocaine only by a phosphonate replacement at the acyl group and by the incorporation of a tether for construction of an immunogenic conjugate has been chosen. Based on their distances from the locus of reaction and their separation from each other, three tether sites were chosen: at the methyl ester for analog 1, the 4'-position of the phenyl group for analog 2, and the tropane nitrogen for analog 3 (Figure 1). The "free TSA" corresponded to the untethered structure 4.

The synthesis of TSA 1 began with the commercially available starting material (-)-ecgonine (Figure 2). Selective alkylation of the carboxylate salt of (-)-ecgonine with 4-azido-1-iodo-butane yielded ester 5 in 78% yield. The absence of epimerization at C-2 was confirmed by ^{1}H-nmr spectroscopy. The base labile and sterically hindered alcohol of alkyl ecgonine 5 reacted smoothly with phenylphosphonic dichloride using the procedure for 1*H*-tetrazole catalysis (14) and addition of methanol provided the phosphonate diester 6 in 89% yield. The tether was elaborated at the azido moiety by reduction to the unstable amine with $P(CH_3)_3$ and acylation with 1,4-^{14}C-succinic anhydride. The hemisuccinate was purified and characterized as the benzyl ester, obtained in 70% yield from 6, and the acid was quantitatively regenerated by catalytic hydrogenolysis. Acid 7 was activated as the *N*-hydroxyphthalimide ester and selectively deesterified at the phosphonate methyl ester with trimethylsilyl bromide (15). The unstable monophosphonate product was immediately coupled to carrier protein to yield TSA-1. The analog:carrier

coupling ratio was 6:1 for bovine serum albumin (BSA) and 15:1 for ovalbumin based on the incorporation of radiolabel into protein. In support of our assignment of structure to the carrier-bound analog, an aliquot of the monophosphonate was coupled to *n*-amylamine to yield the expected amide 8.

Synthesis of TSA-2 required a phenylphosphonic dichloride appropriately substituted at the 4' position for elaboration of a tether (Figure 3). Silylation of 2-(p-bromophenyl) ethanol followed by transmetallation with *n*-butyl lithium, quenching with diethyl chlorophosphate and desilylation provided alcohol 9a in 23% yield. The tosylate of 9a was displaced by azide and transesterification with trimethylsilyl bromide, followed by reaction with oxalyl chloride (16), provided the required phenylphosphonic dichloride 10. Using the tetrazole catalysis method described above, chloride 10 was coupled with ecgonine methyl ester and, after the addition of methanol, the mixed diester 11 was obtained in 25% yield. The tether was elaborated from the azide by a sequence of reactions identical to that employed for TSA-1.

For the synthesis of TSA-3, (Figure 4) *N*-norcocaine was monoalkylated in 75% yield and acid hydrolysis followed by reesterification with acidic methanol provided alcohol 15 in 72% yield. Tetrazole-catalyzed synthesis of mixed phosphonate diester 16 proceeded in 48% yield and the tether was elaborated from the azido moiety as described above.

Generation of anti-cocaine catalytic antibodies

Balb/C mice were immunized with individual analogs conjugated to BSA and high titer antisera were elicited by each antigen. Monoclonal antibodies were prepared by standard protocols (9,17) and hybridomas secreting analog-specific antibodies as determined by an enzyme-linked immunosorbent assay (ELISA) were selected. All IgG anti-analog antibodies were subcloned, propagated in ascites or cell culture flasks and purified by protein A affinity column chromatography. Catalytic antibodies were identified by their capacity to release ^{3}H-benzoic acid from ^{3}H-phenyl-cocaine. The radiolabeled benzoic acid was conveniently partitioned from ^{3}H-cocaine by extraction of the acidified reaction mixture into organic solvent. Hydrolysis of cocaine with commercially available carboxyl esterase provided a positive control and the production of benzoic acid was confirmed by high performance liquid chromatography. A total of nine catalytic antibodies out of 107 anti-analog antibodies were identified from 9 fusions with TSA 1 yielding 6 out of 50 and TSA 3 yielding 2 out of 49. TSA-2 generated eight anti-analog antibodies of which one was catalytic. Catalytic antibodies were further purified by DEAE anion exchange chromatography and they retained activity. All enzymes were inhibited completely by 50 μM free TSA 4 (see below) and the Fab portion of each antibody tested retained catalytic activity; the potent inhibitor of serum esterases, eserine (18) at 1 mM, did not inhibit the activity of any catalytic mAb and 150 μM free TSA 4 did not inhibit the cocaine esterase activity present in serum (results not shown).

Characterization of catalytic antibodies

The rate of hydrolysis of ^{3}H-phenyl-cocaine in the presence and absence of each monoclonal antibody as a function of substrate concentration has been determined. Production of radiolabeled benzoic acid at time points

corresponding to < 5% reaction provided initial rates. A saturation kinetics and obtained a linear Lineweaver-Burk plot for each artificial enzyme has been observed. The first-order rate constants (k_{cat}) and Michaelis constants (K_m) of the nine catalytic antibodies ranged from 0.011 to 2.3 min^{-1} and from 150 to 3000 μM, respectively, as shown in Table 1.

Table 1. Kinetic parameters for the hydrolysis of ^3H-cocaine by Mab's.

Mab	TSA	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/k_o
3B9	1	490	0.11	1100
6A12	1	1020	0.072	880
2A10	1	3000	0.011	420
9A3	1	270	0.015	140
19G8	1	900	0.091	830
15A10	1	220	2.3	23000
12H1	2	150	0.16	1500
8G4G	3	530	0.60	5500
8G4E	3	1200	0.12	1100

Michaelis constant Km; catalytic rate constant, k_{cat}; and spontaneous rate k_o. Assays were performed at the pH that optimized k_{cat}/k_c: in general pH 7.8; for 6A12, pH 7.4; for 2A10, pH 7.0.

The rate acceleration of the most active catalytic antibody, Mab 15A10, was higher and the Michaelis constant lower then those previously reported (9) for Mab 3B9; this corresponds to almost two orders of magnitude improvement in activity at sub-saturating concentrations of cocaine. It has also been reported previously that Mab 3B9 displayed a rate acceleration commensurate with the ratio of K_m to the K_i for free TSA 4. This ratio approximates the affinity of antibody for ground-state relative to transition-state and in the case of Mab 3B9

suggested that the rate acceleration resulted primarily from transition-state stabilization (19). The inhibition constant (K_i) of free TSA 4 for Mab 15A10 to be 0.23 μM has been determined; the rate acceleration of this catalytic antibody (k_{cat}/k_{uncat} = 2.3×10^4) significantly exceeded K_m/K_i (9.6×10^2).

The dissociation constant K_{TSA} for all the catalytic antibodies by competitive inhibition enzyme immunoassay (20) has been determined (CIEIA) as shown in Table 2.

Table 2. Competitive Inhibition Enzyme Immunoassay of catalytic Mab's

Mab(TSA)	K_4 (μM)	K_8 (μM)	K_{13} (μM)	K_{18} (μM)
3B9 (1)	0.01	0.02	3	100
6A12 (1)	0.01	0.01	4	90
2A10 (1)	0.5	3	20	150
12H1 (2)	0.001	0.01	2	60
9A3 (1)	0.05	0.02	-	0.003
19G8 (1)	0.008	0.001	-	0.001
15A10 (1)	0.009	0.003	-	0.0005
8G4G (3)	0.003	0.001	-	0.001
8G4E (3)	0.003	0.0005	-	0.003

Dissociation constants for free TSA 4 and TSA-related amides 8, 13, or 18 were determined for each catalytic Mab by CIEIA through competitive inhibition of Mab binding to the TSA (1, 2 or 3 tethered to ovalbumin) that elicited the Mab.

K_{TSA} determined by CIEIA provides a relative measure of K_i and permits assay at very low concentrations of antibody.

As shown in Figure 1, a log-log plot of k_{cat}/k_{uncat} vs. K_m / K_{TSA} displayed a linear relationship ($r = 0.85$) for 7 of the 9 catalytic antibodies; since K_{TSA} is proportional to K_i, the relationship $k_{cat}/k_{uncat} \cong K_m/K_i$ for Mab 3B9 is likely true for all seven antibodies. Mab 15A10 deviated from this line, as expected since k_{cat}/k_{uncat} exceeded K_m/K_i as described above; Mab 8G4G also apparently deviated as shown. Thus, the rate acceleration for 15A10, and perhaps 8G4G, appears too great to be solely attributed to transition-state stabilization and the participation of chemical catalysis, such as acid-base or nucleophilic catalysis, is likely.

Mab 15A10 was not inhibited by the product of cocaine hydrolysis, ecgonine methyl ester, at a concentration of 1 mM. Benzoic acid did inhibit with a K_i of 250 μM. However, in humans, benzoic acid plasma levels are markedly suppressed by a rapid and nearly complete conversion to hippuric acid (21). It was found that 1 mM hippuric acid did not inhibit Mab 15A10. Also, there was no inhibition from 1 mM benzoyl ecgonine, a prominent metabolite of cocaine in man (22). Inactivation of Mab 15A10 by repetitive turnover was not observed; after 6 hrs, and > 200 turnovers, the k_{cat} remained > 95% of baseline. The presence of minimal product inhibition by ecgonine methylester was fortuitous; heterologous immunization (23) with TSA 1, 2, and 3 and the corresponding 1,2-aminoalcohol analogs of cocaine is planned both for its potential to minimize product inhibition and its capacity to increase the yield of active enzymes.

The rationale for varying the tether sites of TSA to carrier protein (BSA) was to expose unique epitopes and elect catalytic antibodies specific to each immunogen. In order to assess binding specificity, the catalytic antibodies were examined by ELISA with TSA 1, 2, and 3

bound to ovalbumin. Unexpectedly, two groups with broad affinities were identified, a "3B9 group" (Mab's 3B9, 6A12, 2A10, 12H1) that bound all three conjugates and a "9A3 group" (Mab's 9A3, 19G8, 15A10, 8G4G, 8G4E) that bound only TSA-1 and 3.

To estimate the affinities for TSA 1, 2, and 3 within these groups relative K_d's of the corresponding amides 8, 13, and 18 by CIEIA has been determined. As shown in Table 2, CIEIA confirmed the ELISA result, identifying the same two broad groups of catalytic antibodies. The 3B9 group displayed the rank order of affinities: 8 > 13 > 18. The relative K_d for the amide of the TSA that elicited each antibody ranged from 0.01 μM for Mab 3B9 and 6A12 to 3 μM for Mab 2A10. Mab 12H1 derived from TSA 2 showed a greater affinity for the TSA1-related amide 8 (0.01 uM) then for the TSA2-related amide 13 (2 uM). TSA 1 could have elicited Mab 12H1 and the affinities of Mab's 3B9, 6A12 and 2A10 for 13 are also probably sufficient for TSA 2 to have elicited them. The very low affinities of the 3B9 group for the TSA3-related amide 18 suggest that TSA 3 could not have elicited this group.

The 9A3 group showed a distinctly different pattern with very high affinity for TSA1-related amide 8 and TSA3-related amide 18 but virtually none for TSA2-related amide 13. Apparently, TSA-1 or TSA-3 could have elicited every member of this group; TSA-2 could not have elicited any.

To assess the structural diversity of the catalytic Mab's, pcr-cloning and sequencing the variable regions of the heavy and light chains of each antibody were performed. Primers were generally derived from published consensus sequences (24). The 600-700 bp pcr fragment from each reaction was cloned into *p*Bluescript and independently prepared clones were sequenced in both

directions. The deduced primary amino acid structures contained the *N*-terminal amino acid sequences derived from authentic catalytic antibody samples. Amino acid sequencing also provided primers for pcr-cloning of Mab's 2A10 and 15A10. The complementarity determining regions (CDR's) were aligned for comparison (Table 3), and several discrete families of anti-cocaine catalytic antibodies were identified.

Table 3. Deduced amino acid sequences of catalytic antibodies light chain CDR's (Panel A) and heavy chain CDR's (Panel B).

A.

Mab	CDR1	CDR2	CDR3
3B9	RSSRSLLYRDGKTYLN	LMSTRSS	QHFVDYPFT
6A12	RSSKSLLYEDGKTYLN	LMSTRAS	QHFEDYPFT
2A10	RSSKSLLYEDGKTYLN	LMSTRAS	QQFVEYPFT
12H1	RSSRSLLYRDGKTYLN	LMSTRAS	QHFEDYPFT
9A3	RSSTGTI-TTSN-YAN	INNNRPP	ALWYSNHWV
19G8	RSSAGTI-TTSN-YAN	VNNNRPP	ALWYSNHWV
15A10	RSSTGTI-TSDN-YAN	VNNYRPP	ALWYSNHWV
8G4G	RSSSGTI-TANN-YGS	VSNNRGP	ALWNSNHFV
8G4E	KSSQSLLYSDGKTYLN	LVSKLDS	VQGYTFPLT

B.

Mab	CDR1	CDR2	CDR3
3B9	SDYAWT	YIR-HIYGTRYNPSLIS	YHYYGS-AY
6A12	SDYAWY	YIR-HIYGTRYNPSLIS	YHYYGS-AY
2A10	SDYAWN	YIR-YSGITRYNPSLKS	IHYYG-YGN
12H1	SDYAWT	YIR-HIYGTRYNPSLIS	YHYYGS-AY
9A3	-DYNMY	YIDPSNGGIFYNQKFKG	-G-GGLFAY
19G8	-DYNMY	YIDPHNGGIFYNQKFKG	-G-GGLFAY
15A10	-DYNMY	YIDPSNGDTFYNQKFQG	-G-GGLFAF
8G4G	T-YYIY	GMNPGNGVTYFNEKFKN	--VGNLFAY
8G4E	-DHWMH	TIDLSDTYTGYNQNFKG	-R-G--FDY

TSA 1 yielded two structural families, 3B9-6A12-2A10 and 9A3-19G8-15A10. The light chain CDR homology for parings

within the 3B9 family averaged 96%; within the 9A3 family the average was 93%; whereas between these families the average was 14%. The heavy chain CDR homology within the 3B9 family was high with 3B9 and 6A12 identical and 2A10 67% homologous; within the 9A3 family the average heavy chain CDR homology was 88%; but between the 3B9 and 9A3 families the average was 32%. TSA 3 yielded two single-membered families 8G4G and 8G4E. The light chain CDR homology for 8G4G showed 68% homology to the 9A3 group and ≤ 20% homology to the others; 8G4E showed 56% homology with the 3B9 group and ≤ 20% to all others. The heavy chain CDR homology between 8G4G and 8G4E was 24%; for each to the 9A3 group 48% and < 20% to all others. Mab 12H1, derived from TSA-2, showed high homology (96%) to the light chain CDR's of the 3B9-6A12-2A10 group and was identical to the heavy chain CDR's of 3B9 and 6A12.

Example of synthesis of an single Chain Fv Fragment

Single chain Fv fragments for catalytic monoclonal antibody 3B9 have been prepared via the following construction.

Mab 3B9 DNA of V_H and V_L were subcloned by PCR using following primers V_H:

5'TATCCATATGGAGGTGCAGCTGCAGGAGTCTGGACCTGAGCTGGTGAA GCC3'

and

5'ATGGGGGTGTCGGCATGCCTGCAGAGAC3';

and the following primers V_L:

5'CCCCATGGATATTGTGATGACCCAGGAT3'

and

5'TAACTGCTCGAGGGATGGTGGGAA3'.

DNA of V_L was digested by *Nco I* and *Xho I* and introduced into pET20b (Novagen). DNA of V_H was digested by *Nde I* and

SphI, and introduced into pUC18 containing a following linker sequence:
(*SphI*)-CATCCGGAGGCGGTGGCTCGGGCGGTGGCGGCTCGGGTGGCTCTGC-(*NcoI*).

This plasmid was digested by NdeI and NcoI, and introduced into pET20b containing V_L DNA. Then, this plasmid was digested by *Xho I* and a following sequence that codes flag sequence was introduced; TCGATTACAAGGACGACGATGACAAGC. The resulting plasmid was transformed into BL21(DE3) pLysS. Cells were grown in LB medium at 37°C. At an OD_{550} of 0.6 IPTG was added to a final concentration of 2mM, and the cells were further grown for 2 hrs. before harvest. The cells were suspended in 20 of culture volume of binding buffer (5mM imidazole/0.5M NaCl/20mM Tris-HCl, pH 7.9)/6M Urea, disrupted by freezing and thawing and removed debris by centrifugation (10000g x 20 min). Supernatant was applied to HistBind Resin Column (Novagen) and eluted with 6M urea/1M imidazole/0.5M NaCl/20mM Tris-HCl pH 7.9.

Elisa analysis of the resulting single chain Fv fragment demonstrated binding activity. Enzymatic activity was confirmed by the release of the ^3H benzoic acid from the ^3H phenyl-cocaine.

EXPERIMENTAL DISCUSSION

The clinical application of a catalytic antibody against cocaine relies on a kinetic argument since a 100 mg dose of cocaine if antagonized solely by antibody binding would require 25 g of antibody (assuming an antibody MW of 150 kD and 2:1 cocaine:antibody stoichiometry). Active immunization with cocaine tethered to an immunoconjugate would be unlikely to provide more than a few percent of this requirement (25). Polyclonal gamma globulin can be administered in doses of this magnitude

but clearly only enzymatic turnover reduces the antibody requirement to a practical magnitude and, most importantly, allows for the burden of repetitive self-administration - the hallmark of addiction.

The optimization of an anti-cocaine catalytic antibody which greatly reduces the cost per dose can be approached through improved analog design, large scale antibody selection (26) and antibody mutagenesis (27). Mab 15A10 and 8G4G are the preferred candidates for optimization since they are the most active catalytic antibodies; they are structurally distinct (see below); and Mab 15A10, and possibly 8G4G, could already manifest some element of chemical catalysis. The failure of decades of effort to identify classical receptor blockers of cocaine, together with the compelling nature of the cocaine problem, justify an exhaustive strategy employing all three approaches. One impediment to this effort is the limited diversity of the antibodies elicited by a given analog. Clearly, antibody diversity is not necessary if, by chance, a single class of antibodies ultimately yields a member with the desired kinetic parameters. However, the capacity of a given antibody to be optimized to specification cannot be predicted due to the scarcity of structural data on catalytic antibodies. The generation of a diverse group of anti-cocaine catalytic antibodies should improve the prospects for successful optimization whether through repetitive large-scale hydridoma preparation or through mutagenesis.

Using the tetrazole catalysis method for phosphonate ester synthesis, three transition-state analogs of cocaine hydrolysis were synthesized. The core phosphonate monoester structure was identical in each and only the tether sites varied. All three elicited catalytic antibodies and a competitive ELISA and CDR sequencing were used to define functional and structural

groupings, respectively.

A comparison of the CDR's of the active antibodies delineated four discrete non-overlapping families that were elicited specifically by TSA 1 (3B9-6A12-2A10 and 9A3-19G8-15A10) and TSA 3 (8G4G and 8G4E). TSA 2 yielded one antibody highly homologous to the 3B9-6A12-2A10 family from TSA 1 and without homology to the antibodies derived from TSA 3. These structural families overlapped in part with two broad groups defined by a CIEIA method in which amides 8, 13, and 18 (representing TSA 1, 2 and 3, respectively) inhibited the binding of each catalytic antibody to its eliciting TSA.

One group defined by CIEIA consisted of Mab's 3B9, 6A12, 2A10 and 12H1. This group displayed high affinity for 8, moderate affinity for 13 and very low affinity for 18. All of the highly homologous members of this group could have been elicited by TSA 1; the one antibody derived from TSA 2, Mab 12H1, bound TSA1-related amide 8 with even greater affinity than TSA2-related amide 13. Nonetheless it is possible that most if not all of the group could have been elicited by TSA 2 since the range of affinities for 13 in this group overlapped with the range of affinities for the amides of the TSA's that elicited each antibody. In contrast, the very low affinity of 18 for every member of this group suggests that TSA 3 could not yield any member of the group. A strategy to obtain catalytic antibodies against cocaine based only on a TSA tethered at the tropane nitrogen (28) would fail to identify this group of antibodies.

The second group defined by CIEIA consisted of five catalytic antibodies from three structural families: 9A3-19G8-15A10 derived from TSA 1; 8G4G and 8G4E from TSA 3. These five antibodies displayed equally high affinity for amides 8 and 18 and in principle either TSA 1 or 3 could

have elicited every catalytic antibody in this group. That TSA 1 and 3 did not yield members of a common structural family may reflect the inadequacy of a sample size averaging 3 fusions per analog. None of the five antibodies could have been obtained with TSA 2 and thus three of the four structural families would not have been identified with this conjugate.

TSA 1 elicited the most active catalytic antibody, Mab 15A10. Moreover, based on the high affinity of amide 8 for all nine catalytic antibodies, TSA 1 could plausibly have elicited every antibody described. This result was unexpected but not a definitive endorsement of TSA 1 as the preferred analog. With more aggressive screening, TSA 2 or 3 may ultimately yield a more active antibody not recognized by TSA 1.

Clearly, the failure of a TSA (e.g. TSA 2) to bind to a catalytic antibody (e.g. 15A10) derived from an alternate immunogenic conjugate confirms that the location of the tether limits the catalytic antibodies produced and supports varying the site of attachment to carrier protein. Exhaustive screening of hybridomas from TSA 1, 2 and 3 and detailed structural studies of the catalytic antibodies elicited may clarify the rules for analog construction. The pursuit of high activity anti-cocaine catalytic antibodies provides a compelling justification for this effort.

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SECOND SERIES OF EXPERIMENTS

Introduction

Cocaine overdose, a potentially fatal syndrome, has long defied development of antagonists. To provide a new approach, a high activity catalytic antibody was elicited using a transition-state analog for the hydrolysis of cocaine to non-toxic products. This antibody protected rats from cocaine-induced seizures and sudden death in a dose-dependent fashion. Consistent with accelerated catalysis, the hydrolysis product ecgonine methyl ester was increased > 10-fold in plasma; a non-catalytic anti-cocaine antibody did not reduce toxicity. This artificial cocaine esterase is the first rationally designed cocaine antagonist and the first catalytic antibody with potential for medicinal use.

Cocaine is presently abused in the United States by approximately two million hardcore addicts and over four million regular users (1). The acute toxicity of cocaine overdose frequently complicates abuse and the potential medical consequences of this syndrome include convulsions and death (2). Despite decades of effort, however, no useful antagonists to cocaine have been found. This failure is due, in part, to the drug's unique mechanism of action as a competitive blocker of neurotransmitter re-uptake (3). Thus, cocaine's blockade of a dopamine re-uptake transporter in the central nervous system (CNS) is hypothesized to cause reinforcement (4) and the difficulties inherent in blocking a blocker appear to have hindered the development of antagonists for addiction. For cocaine overdose this problem is compounded by the binding of cocaine at high concentrations to multiple receptors in the CNS and cardiovascular systems. For instance, blockade of serotonin-reuptake transporters contributes to cocaine-

induced convulsions (5,6); dopamine-reuptake blockade (6) and dopamine D_1 receptor binding (7) contribute to lethality; and blockade of norepinephrine-reuptake transporters, as well as blockade of cardiac myocyte Na^+ channels and other ion transporters, contributes to arrhythmias and sudden death (8). Thus, cocaine overdose may well pose an insurmountable problem for the classical receptor-antagonist approach.

These difficulties in developing antagonists for cocaine abuse led to a new approach - to intercept cocaine with a circulating agent thereby rendering it unavailable for receptor binding. An antibody is an obvious choice for a circulating interceptor but, as noted in the original 1974 report on anti-heroin antibodies, the stoichiometric binding of the drug effectively depletes antibody (9). To overcome the limitations of binding, catalytic antibodies were developed - a novel class of artificial enzyme (10) - with the capacity to bind and degrade cocaine, release product and become available for further binding (11). Since degradation of cocaine at its benzoyl ester yields non-toxic products, ecgonine methyl ester (12) and benzoic acid (13) (Figure 28A), a phosphonate monoester transition-state analog for benzoyl ester hydrolysis (TSA-I, Figure 28B), was synthesized and with it elicited the first catalytic antibodies to degrade cocaine <u>in vitro</u> (11).

The catalytic activity of these antibodies was insufficient to demonstrate a biologic effect but through repetitive hybridoma preparation with the reagent TSA-I, Mab 15A10, an antibody 100-fold more potent at sub-saturating concentrations of cocaine (14) was generated. This antibody is the most potent artificial cocaine esterase to date with a Michaelis constant of 220 μM, a turnover rate of 2.3 min^{-1}, and a rate acceleration of 2.3 x 10^4. The antibody retained >95% of its activity after

>200 turnovers and product inhibition, a frequent impediment to useful antibody catalysis (15), was not observed for the alcohol product ecgonine methyl ester at concentrations up to 1 mM. Although Mab 15A10 was inhibited in vitro by benzoic acid (Kd ~ 250 μM), this acid is rapidly cleared from plasma through coupling to glycine (13,16) and the adduct, hippuric acid, was not an inhibitor in vitro at a concentration of 1 mM. Thus, Mab 15A10 possesses several characteristics essential for a practical in vivo catalyst.

Using Mab 15A10, the antibody-catalyzed degradation of cocaine was tested to see if it could block the acute toxicity of cocaine overdose in rat. The toxicity of cocaine can vary significantly among individuals depending on endogenous catecholamine levels and this likely explains the variably increased incidence of sudden death in restrained animals (17) and agitated patients (18). In previous work (19), catecholamine levels were standardized through intravenous infusion in conscious, unrestrained animals and, for continuously infused cocaine (1 mg/kg/min), found that the LD_{50} was 10 mg/kg and the LD_{90} was 16 mg/kg.

Using this method (20), animals pretreated with Mab 15A10 (21) showed a significant ($p<0.001$) dose-dependent increase in survival to an LD_{90} cocaine infusion (Figure 29). Four of five animals receiving antibody at 15 mg/kg and all of five receiving antibody at 50 mg/kg survived. In contrast, all eight rats not treated with Mab 15A10 expired before the cocaine infusion was complete. In the animals not treated with Mab 15A10, the mean cocaine dose at death was 7.5 ± 0.6 mg/kg, whereas the five treated with antibody at 5 mg/kg expired at a mean cocaine dose of 8.2 ± 1.0 mg/kg and the single non-survivor in the group treated with antibody at 15 mg/kg expired at 15.9 mg/kg of cocaine.

To further quantify the protective effect of the catalytic antibody, the 15A10 (100 mg/kg) and control groups were overwhelmed with intravenous cocaine continuously administered at 1 mg/kg/min until all animals expired (Figures 30A and 30B). The dose of cocaine at seizure averaged 9.48 mg/kg for saline controls and 32.5 mg/kg for animals treated with Mab 15A10 ($p<0.01$) (Figure 30A). The mean lethal dose of cocaine was also increased over 3-fold, from 11.5 mg/kg of cocaine for controls to 37.0 mg/kg for the Mab 15A10 group ($p<0.01$) (Figure 30B).

Simple binding was an unlikely explanation for the effectiveness of Mab 15A10 since stoichiometric binding of cocaine would be expected to shift the dose-response to cocaine by < 1 mg/kg. However, to exclude this possibility, the action of a binding antibody, Mab 1C1, was tested at an equal dose. Mab 1C1 was elicited by immunization with TSA-I, but the antibody is not catalytically active since it binds free TSA and cocaine with comparable affinity (22). As expected, Mab 1C1 was ineffective in blocking cocaine-induced convulsions or death (Figures 30A and 30B).

To demonstrate <u>in vivo</u> catalysis, the plasma concentrations of cocaine hydrolysis products in the 15A10 and control groups were measured by previously developed high-pressure liquid chromatography (HPLC) method (23). The 15A10 group showed a >10-fold increase in ecgonine methyl ester (24) compared to either the saline ($p<0.001$) or the Mab 1C1 ($p<0.01$) control groups (Figure 30C). As expected based on its rapid metabolism (13,16), plasma benzoic acid concentrations were not significantly elevated in the 15A10 group (3.85 ± 0.89 μM) compared to the saline control group (2.36 ± 1.05 μM). Consistent with specific catalysis at the benzoyl ester, the plasma concentration of the methyl ester hydrolysis

product, benzoyl ecgonine (Figure 28A), was not significantly increased in the Mab 15A10 group (7.68± 1.07 mM) compared to saline control (5.47± 1.01 μM).

Plasma cocaine concentrations in 15A10 and control groups were measured at death by HPLC (23) in order to confirm that Mab 15A10 conferred resistance to cocaine toxicity through a pre-receptor mechanism. A marked elevation of plasma cocaine would be expected if Mab 15A10 acted at or after the binding of cocaine to its receptors. In contrast, plasma cocaine concentrations at death were not significantly different between 15A10 and control groups (Figure 30D), as expected for a pre-receptor effect and consistent with protection from toxicity through catalyzed degradation of cocaine.

The present study provides a proof of the concept for the use of circulating catalytic antibodies to block the toxic effects of cocaine. The incidence of cocaine overdose in the United States is approximately 80,000 cases per year and cocaine-related deaths exceed 3,000 per year (1). An anti-cocaine catalytic antibody could be a useful therapeutic for patients manifesting serious complications of overdose such as seizures and arrhythmias. Mouse monoclonal 15A10, the first catalytic antibody with potential for medicinal use, is a suitable candidate for mutagenesis to further improve kinetics(25) and protein engineering to enhance human compatibility (26). Assessment of Mab 15A10 and more active homologs in an animal model based on antibody post-treatment of cocaine toxicity would precede human trials.

Since the original report on anti-cocaine catalytic antibodies (3), others have described variations on the concept of intercepting cocaine before the drug reaches its receptors. For example, intraperitoneal administration of the enzyme butyrylcholinesterase was

shown to inhibit toxicity due to intraperitoneal cocaine in mouse (27). Also, non-catalytic anti-cocaine antibodies were shown to diminish cocaine-induced psychomotor effects and reinforcement in rat (28). However, catalytic antibodies are likely to be longer-lived in plasma than natural enzymes and, in contrast to typical antibodies, not susceptible to depletion by complex formation with cocaine. Thus, catalytic antibodies have the unique potential to treat both the acute and chronic aspects of cocaine abuse and, as a result, practical experience with acute overdose can provide a foundation for the treatment of chronic addiction.

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(0.8 μg/min)] were infused intravenously with co-infusion of cocaine at one mg/kg/min for 16-min. HPLC measurements of catecholamines levels(9) at baseline and at the time of cardiopulmonary arrest were found not to be significantly different between groups ($p>0.05$).

21. Hybridoma 15A10 was seeded in a Fibra Cel cell support matrix (Cellagen Plus bioreactor, New Brunswick Scientific Co, New Brunswick, NJ) continuously perfused with RPMI 1640 (GIBCO) medium. Perfusate was concentrated with a prep. scale 10K 6 sq. ft. cartridge (Millipore) and subjected to Protein G chromatography to yield Mab 15A10 >90% pure by SDS-PAGE chromatography. Catalytic activity was comparable to that previously described[14] and was completely inhibited by free TSA (50 μM). Endotoxin levels were < 0.1EU/ml by QCL - 1000 quantatitive chromogenic LAL assay.

22. Mab 1C1 was obtained from the original hybridoma preparation with TSA-I as described(14). For Mab 1C1, the cocaine IC_{50} was 30 μM by inhibition of ^{3}H-cocaine binding (31 mCi/mmol, New England Nuclear, Waltham, MA) with cold cocaine 0-1000 μM in phosphate buffered saline (pH 7.4). Bound radiolabel was separated from free by gel filtration chromatography using standard methods: D. W. Landry, M. Reitman, E. J. Cragoe, Jr., and Q. Al-Awqati. J. Gen. Physiol. 90:779, (1987).

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